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Slipping pumps or proton leaks in oxidative phosphorylation

The local anesthetic bupivacaine causes slip in cytochrome *c* oxidase of mitochondria

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Evidence is presented to show that the local anesthetic bupivacaine causes slip in the mitochondrial proton pump cytochrome *c* oxidase.

Oxidative phosphorylation; Uncoupling; Mitochondria; Local anesthetic; Proton pump

1. INTRODUCTION

Recently, we found that the local anesthetic bupivacaine inhibits ATP synthesis by uncoupling of oxidative phosphorylation in mitochondria, but without increase in the proton permeability of the mitochondrial membrane [1], in contrast to the classical chemiosmotic concept [2]. In the presence of the amphiphilic anions like 1-anilinonapthalene-8-sulfonate (ANS), however, bupivacaine caused uncoupling accompanied by an increase of the proton permeability or leak of the membrane [1]. Uncoupling without association with a proton leak has been called decoupling [3,4], and is interpreted to be due to the slip of proton pumping complexes, such as respiratory chain and ATP synthase [3-7]. Since it would be very important to know whether decoupling is really due to a slip or not, we examined the effect of bupivacaine on the proton pumping activity of cytochrome *c* oxidase, and found that bupivacaine alone causes slip, but in combination with ANS it causes proton leak in the mitochondrial membrane.

2. MATERIALS AND METHODS

Mitochondria were isolated from the liver of adult male Wistar rats. The incubation medium, in a total volume of 1.5 ml, consisted of 150 mM KCl, 0.67 mM EDTA and 1 mM Hepes, pH 7.4. Rotenone (0.27 μg/mg mitochondrial protein), antimycin (0.53 μg/mg mitochondrial protein), oligomycin (0.53 μg/mg mitochondrial protein), and *N*-ethylmaleimide (53 nmol/mg mitochondrial protein) were always present in the mitochondrial suspension. The reduction of O_2 by cytochrome *c* oxidase upon addition of 1.33 mM ferrocyanide was monitored polarographically at 25°C as described previously [9]. The concentration of mitochondria was 5 mg protein/ml. Bupivacaine was a generous gift from Fujisawa Pharmaceutical Industry Co, Osaka, Japan. In the experiments of Fig. 2 valinomycin at 0.53 μg/mg mitochondrial protein was present in the incubation medium. The movement of protons was monitored with a glass electrode connected with a pH meter, Horiba F-7. The extent of proton extrusion (ΔH^+) was determined as described in the text.

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3. RESULTS AND DISCUSSION

Uptake of O_2 started upon addition of ferrocyanide to a suspension of rat-liver mitochondria that had been inhibited in all functions related to oxidative phosphorylation except that of cytochrome *c* oxidase. The rate of oxygen uptake (V_{ox}) was accelerated by addition of a protonophore uncoupler like SF 6847 as a result of the leakage of protons across the membrane caused by the uncoupler [8]. The tertiary amine local anesthetic bupivacaine at 1 mM also accelerated V_{ox} and this effect was potentiated by 40 μM ANS, as had been observed with mitochondria energized by succinate [1].

Fig. 1 summarizes the dependence of V_{ox} on the concentration of bupivacaine either in the presence or absence of 40 μM ANS. In the absence of ANS, bupivacaine increased V_{ox} and the maximal rate of respiration was attained at about 1 mM. The effect of bupivacaine was enhanced in the presence of 40 μM ANS and V_{max} was attained at about 1 mM.

To examine the efficiency of proton pumping of cytochrome *c* oxidase, we next measured the external pH change of a mitochondrial suspension under similar conditions to those described above, but in this case valinomycin was added for charge compensation [9]. Upon addition of ferrocyanide, consumption of pro-

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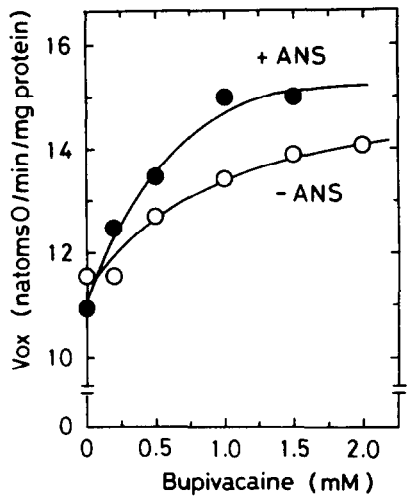

Fig. 1. Dependence of rate of oxygen consumption (V_{ox}) due to the oxidation of ferrocyanide by cytochrome *c* oxidase in rat-liver mitochondria on the concentration of bupivacaine with and without 40 μM ANS.

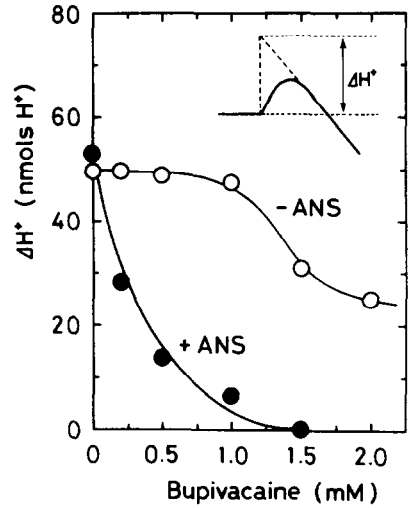

Fig. 2. ANS-dependent inhibitory effect of bupivacaine on the proton extrusion through cytochrome *c* oxidase.

tons due to reduction of oxygen to water mediated by cytochrome *c* oxidase was observed after an initial rapid burst of protons [9]. The rapid burst was completely abolished by SF 6847, as reported previously for another weakly acidic uncoupler [9]. The rate of electron transport from ferrocyanide to oxygen was not increased by the addition of uncoupler, presumably because of the presence of valinomycin and a high concentration of K^+ ions.

Bupivacaine up to 1 mM did not have an effect on the extent of proton movement elicited by ferrocyanide, although at this concentration it accelerated V_{ox} almost maximally, in the absence of valinomycin (cf. Fig. 1). In contrast, bupivacaine at 1 mM in the presence of 40 μM ANS completely abolished the rapid extrusion of protons as observed with SF 6847. The dependence of the proton extrusion by cytochrome *c* oxidase on the concentration of bupivacaine, either in the absence or presence of 40 μM ANS, is summarized in Fig. 2. The extent of proton ejection (ΔH^+) was determined as the amount of protons ejected after extrapolation of the steady-state proton uptake to the point of ferrocyanide addition. In the absence of ANS, bupivacaine up to 1 mM did not show any effect on the proton pumping. Only at higher concentrations bupivacaine caused some decrease in ΔH^+. In contrast, in the presence of 40 μM ANS, bupivacaine caused a strong decrease in proton extrusion depending on the concentration of bupivacaine, and full inhibition was observed at 1.5 mM bupivacaine (cf. Fig. 1).

Thus, we have found that bupivacaine at concentrations that stimulate electron transport maximally have no apparent effect on the number of protons extruded by the pumping activity of cytochrome *c* oxidase. In contrast, if the amphiphilic anion ANS is present, the same concentrations of bupivacaine cause complete loss of proton pumping. We interpret these findings as follows. Bupivacaine alone causes slip in the cytochrome *c* oxidase pump. As long as the activity of the remaining coupled pumps is high enough to compensate for the intrinsic proton permeability of the membrane, these pumps will be able to generate the maximal proton gradient across the membrane. However, the electron transport of the uncoupled (slipping) pumps will contribute to the overall oxygen uptake to an increasing extent. Only when the pumping activity of the coupled pumps falls below the level required to compensate for the intrinsic proton permeability of the membrane, further addition of bupivacaine will cause a decrease in the extent of observable proton pumping.

In the combination of bupivacaine with ANS a different situation arises. This makes the mitochondrial membrane more leaky for protons, just like weakly acidic uncouplers do [8]. As a result of this, the magnitude of the electrochemical gradient of protons across the membrane will decrease and this in turn will cause an increase in electron transport, because the opposing force has decreased. The partial loss of the proton extrusion at concentrations higher than 1.5 mM of bupivacaine in the absence of ANS could be the result of a slight leak of protons.

To our knowledge, this would be the first direct demonstration that a decoupler really causes slip in the proton pumps of mitochondria, although the existence of such a behavior has been predicted [5,6]. There is a fundamental difference between the two types of uncoupling of oxidative phosphorylation: The same degree of coupling in mitochondria may be reached either by controlled leak of protons across the membrane or by controlled slip in the pumps. The important physiological difference between the two situations is that they allow independent modulation of respiration and phosphate potential.

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